Detection of Mutations in EGFR in Circulating Lung-Cancer Cells

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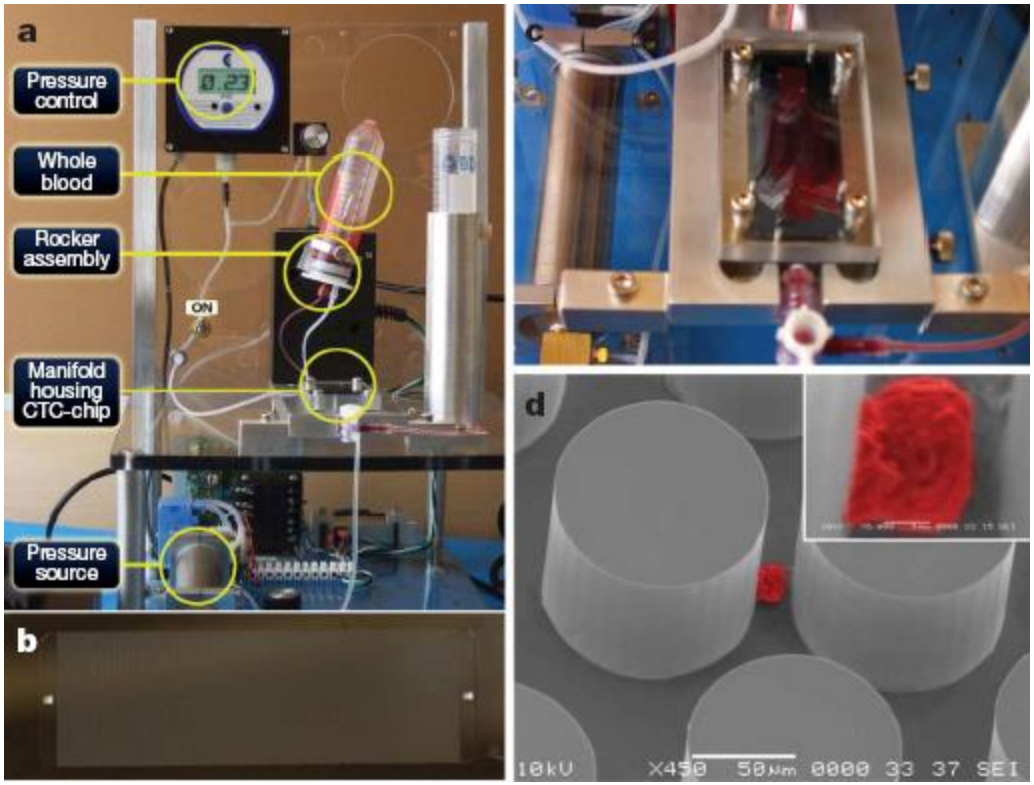
Cancer Treatment: Drug Resistance

- Mutations acquired during treatment → drug resistance
  - Non-small-cell lung cancer: EGFR activating mutations
  - Tyrosine kinase inhibitors eg gefitinib and erlotinib
  - Relapse within 1 year: acquisition of secondary EGFR mutation T790M
- Invasive biopsies provide insufficient material for molec analysis and cannot be sampled repeatedly
- Alternative biomarkers in the blood
  - Cell-free DNA – insensitive detection of tumor growth
- Molecular characterization of circulating tumor cells (CTCs)

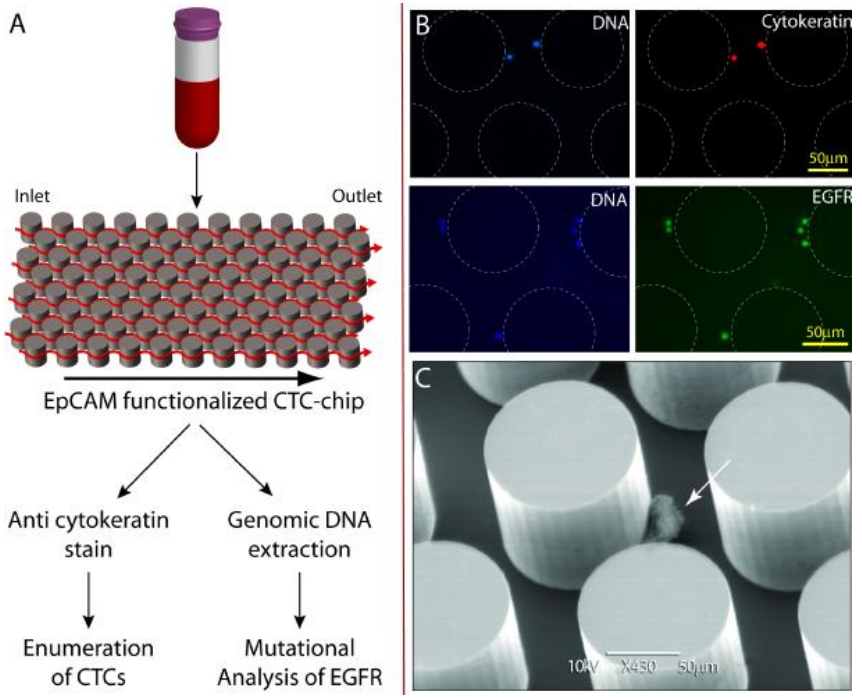
CTC-chip

- Isolate, quantify, analyze CTCs
- Blood flows past EpCAM-coated microposts
- Optimize laminar flow conditions → high yield and purity, reproducible
- Correlated with disease progression



CTC Identification and Enumeration by Fluorescence Microscopy

- Cells fixed on CTC-chip
- Fluorescence Staining
  - Hoeschst →DNA content
  - Cytokeratin →epithelial cells
  - CD45 →leukocytes
- Identify CTCs based on cell size, morphology, and fluorescence



Supplementary figure 1: CTC-chip analysis of blood specimens from NSCL patients

EGFR Mutational Analysis

- Allele-specific mutation detection by Scorpion Amplified Refractory Mutation System (SARMS) in RT-PCR
- Scorpion primer:
  - Quenched fluorophore, gene-specific probe region, and primer to WT or mutant allele
- Primers anneal to DNA
  - Fluorophore still quenched
- Denaturation
  - Self-association of Scorpion primer → dissociation of quencher

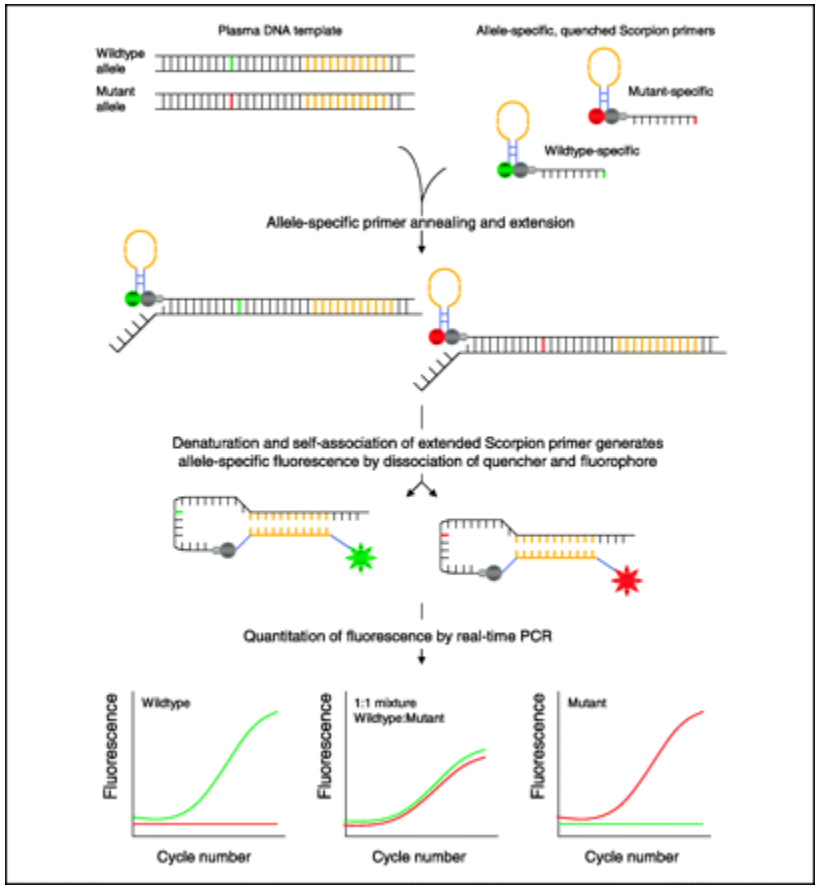


Image from Clinical Cancer Research July 2006*12;* 3875

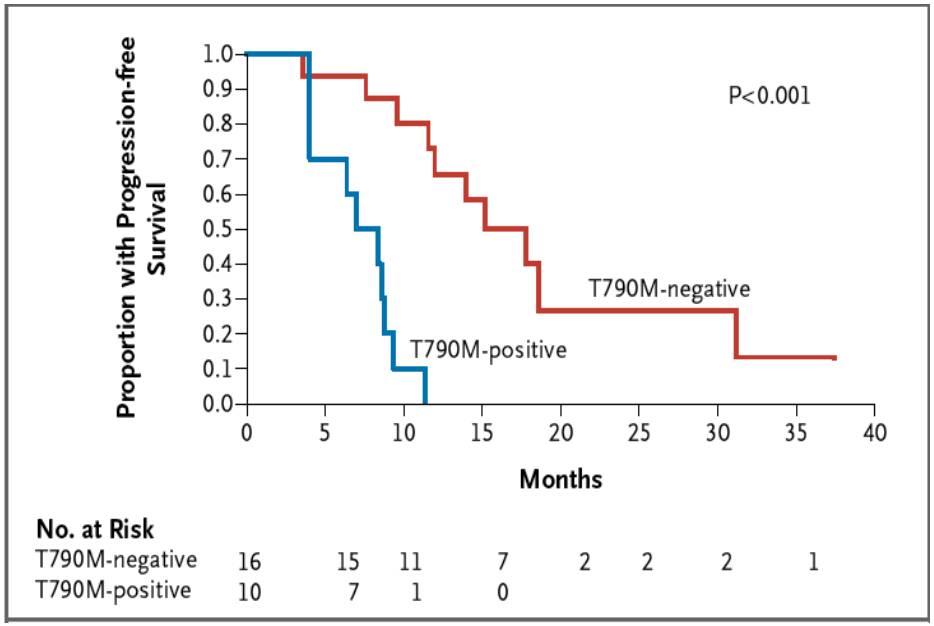
Results

- Identified circulating tumor cells
- Detected EGFR mutations in tumors
- Tumor genotyping over time

Identifying CTCs

- Patients:
  - 23 EGFR mutant tumors
  - 4 WT EGFR tumors
  - 10 mL blood sample
  - Radiographic measurements performed close to time of CTC analysis
- Median of 74 CTCs/mL (mean, 133; range, 5-771)
- Quantity of CTCs did not correlate with tumor volume as assessed by radiographic measurements

Detection of EGFR mutations in tumors

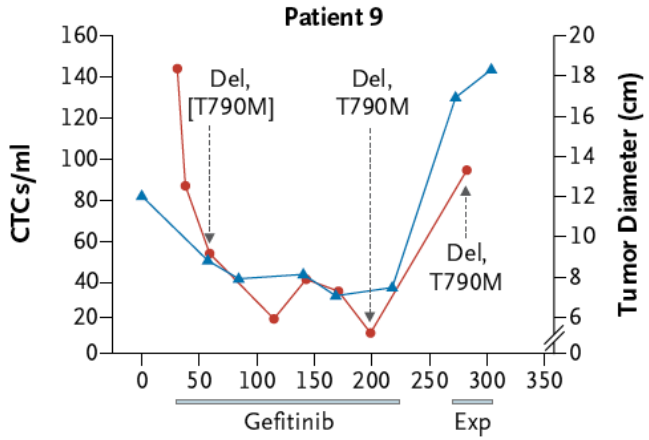


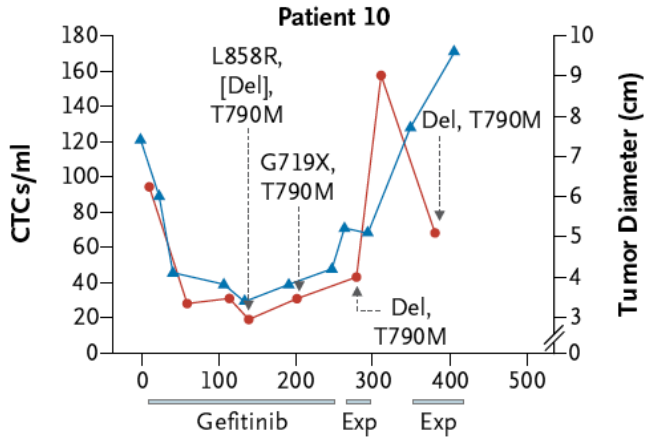
- Mutations:
  - Exon 19 mutations (“del”)
  - L858R missense mutation
  - T790M mutation -> confers resistance to gefitinib and erlotinib
- T790M mutation in pre-treatment tumor samples
  - 10/26 patients
  - High number of amplification rounds

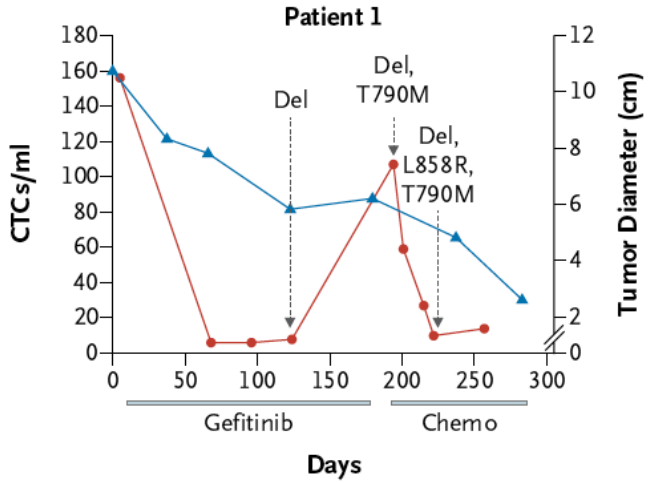
- pre-treatment T790M mutation correlated with progression-free survival
  - 7.7 months vs. 16.5 months

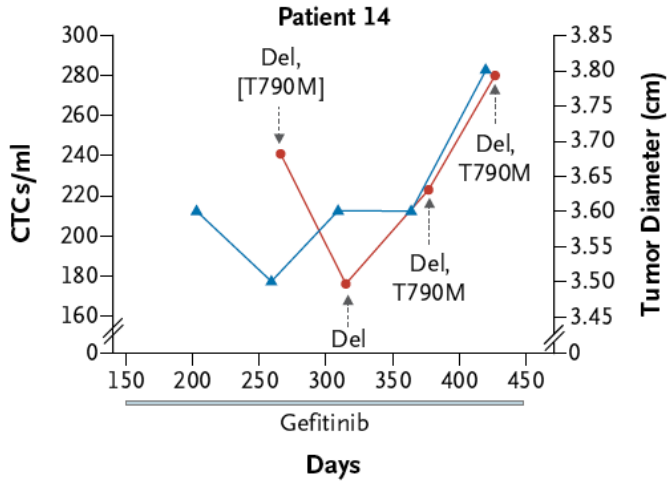
CTC count over time

- Look at effect of treatment on CTCs





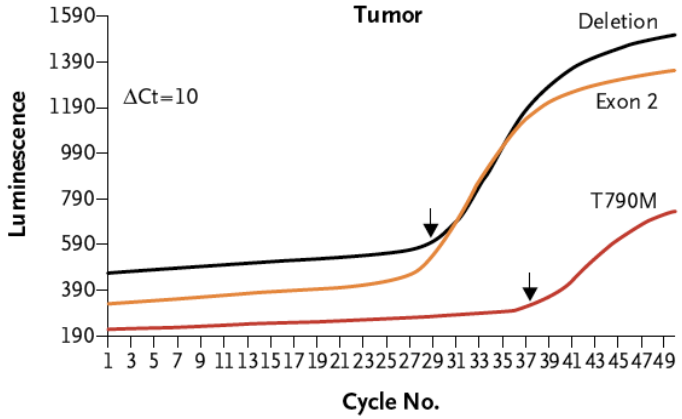


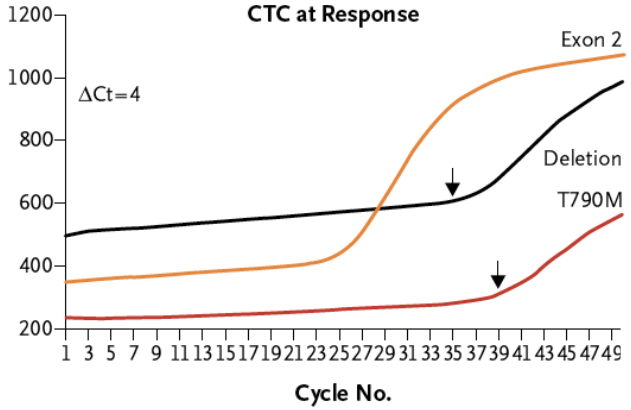


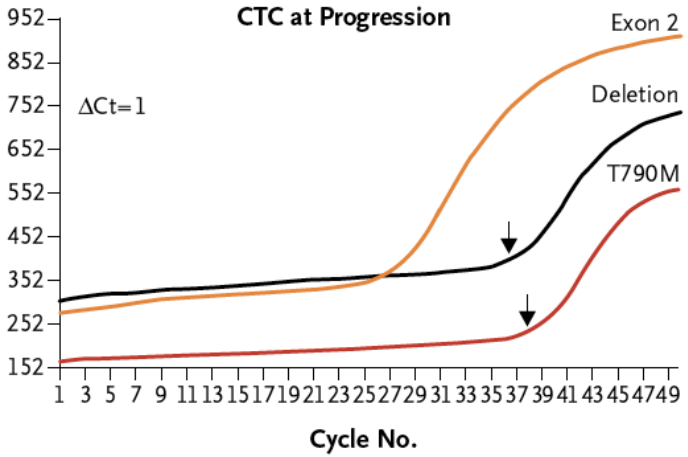
CTCs per mL Tumor size [ ] low allele frequency

Patient 9

B







Treatment-induced genotype shift

Significance

- CTC count may be a good measure of tumor metastasis and invasiveness
  - Better predictor than simply tumor size
- Useful for tumor sub-typing
  - Pre-existence of T790M mutation is a predictor of response to gefitinib/erlotinib treatment
- Track tumor genotypes over time
  - Cancer treatment is a selective process
  - Might aid medical treatment decisions

Limitations and Future Directions

- SARMS limited to known mutations
  - Cheaper/faster sequencing
- Not high-throughput
  - Combine with:
    - Microfluidic DNA isolation
    - Microfluidic PCR
- Application to other cancers with well-characterized mutations

Sources

- Maheswaran, *et al.*, "Detection of Mutations in EGFR in Circulating Lung-Cancer Cells" NEJM 2008.

- Whitcombe D, Theaker J, Guy SP, Brown T, Little S. Detection of PCR products using self-probing amplicons and fluorescence. Nat Biotechnol 1999;17:804-7.

- Nagrath S, Sequist LV, Maheswaran S, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature 2007;450:1235-9.

Validate SARMS assay

- Designed to detect several mutations in EGFR
  - Exon 19 mutations (“del”)
  - L858R missense mutation
  - T790M mutation -> confers resistance to gefitinib and erlotinib
- Compared SARMS and sequencing results
  - Identified the same mutation in 25/26 EGFR-mutant tumors

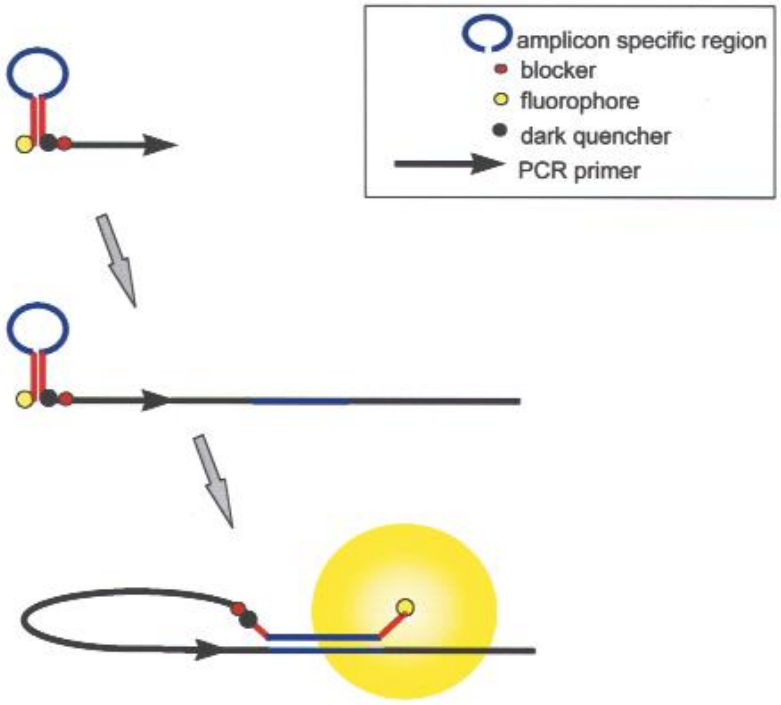


Image from Whitcombe, et al.1999

Comparison of CTC analysis and free plasma DNA analysis

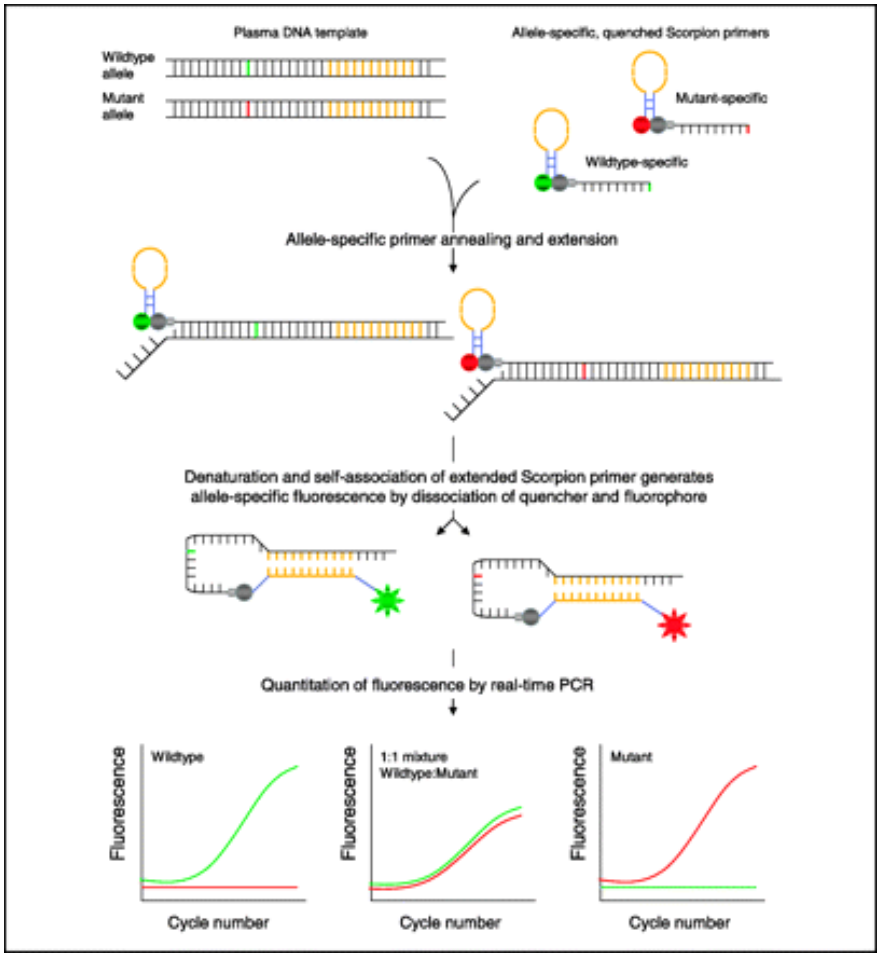
- 18 patients
  - CTCs
  - Free plasma DNA

- 12 patients for sensitivity calculation
  - Primary tumor sample
  - CTCs
  - Free plasma DNA

	EGFR mut	Sensitivity
CTCs	17	92%
plasma	7	33%

SARMS

- Allele-secific primers compose of specific fluorophore, stem, gene-specific probe region, quenchen, blocker molecule, and primer to WT or mutant
- PCR with Scorpion primers specific to WT or mutant alleles
- Allele-specific extension



Statistical Analysis

- Statistical analysis on
  - CTC quantity vs tumor burden
  - Mutations identified in different populations
  - Baseline T790M vs progression-free survival

Patients and Clinical Specimens

Table 1. Detection of Circulating Tumor Cells in Patients with Non–Small-Cell Lung Cancer.*

Patient No. and *EGFR* Mutation Status	Sex	Age	Histologic Features	Time since Diagnosis	Previous Systemic Therapy†	Tumor Burden‡	Circulating Tumor Cells§
		yr		*mo*		*cm*	*no. per ml*
EGFR mutation present							
1	M	58	Adeno	3.2	None	19.8	156
2	M	55	Adeno	14.4	C, E	2.4	50
3	F	66	Adeno	18.2	G, C	19.5	9
4	M	59	Adeno/BAC	20.7	G	2.0	771
5	M	57	Adeno	10.8	C	1.5	152
6	F	74	Adeno	18.3	E	9.8	5
7	M	64	NSCLC	13.1	G, E, C	4.6	196
8	F	70	Adeno	1.4	None	5.8	175
9	F	63	Adeno	0.9	None	28.6	143
10	F	66	Adeno/BAC	1.3	None	8.2	112
11	F	74	Adeno/BAC	4.8	G	4.5	74
12	F	62	Adeno/BAC	56.8	G, E, O	7.2	9
13	M	27	Adeno	9.9	G	4.1	47
14	F	55	Adeno	11	G	5.2	241
15	F	70	Adeno/BAC	54.7	G, C	30.2	31
16	M	53	Adeno/BAC	9.2	E	7.0	49
17	F	60	Adeno/BAC	97.0	G	4.3	103